A METHOD FOR MEASURING A PATIENT'S ABILITY TO METABOLISE CERTAIN DRUGS

The present application relates to an assay method for monitoring metabolism of certain drugs in an individual. More particularly, the invention relates to a method for determining the presence of point mutations in isoforms of cytochrome P450, which point mutations are known to affect the isoforms' abilities to metabolise said drugs. The invention also relates to primers and diagnostic kits that are suitable for carrying out the invention.

Technical background

All reference cited in the following description are incorporated into the disclosure by reference.

Single nucleotide variations have been estimated to occur in a frequency of approximately one out of thousand nucleotides in the human genome (Cooper et al., J. Hum. Genet. (1985) 69:201). Many of these mutations may not give rise to a phenotype but a great number of the genetic diseases known to date are caused by single nucleotide polymorphisms. As a consequence, detection of single nucleotide mutations in specific genes will become of increasing interest in order to understand the ethiology of many genetic diseases.

Drug metabolism involves enzymes that either oxidise (phase I) or conjugate (phase II) xenobiotics. The major route of phase I drug metabolism is maintained by a group of enzymes termed cytochrome P450 which are located in the endoplasmatic reticulum primarily in the liver (Linder et al., Clinical Chemistry (1997) 43:254). Cytochromes P450 (CYP) are comprised by a super gene family of mixed function oxidases that metabolises a large number of xenobiotics including drugs. Thirty or more of these enzymes have been characterised in the human so far, each with distinct catalytic specificity and unique regulation. Because of the diversity of these enzymes, they have been subdivided into subpopulations or isoforms based on their sequence homology, The

polymorphism of the catalytic abilities of these enzymes result in the appearance of different phenotypes with differential capacities to metabolise drugs. Extensive metabolism (EM) of a drug is characteristic of the normal population and represents the wild-type allele, poor metabolism (PM) is due to poor or no catalytic capacity by a specific enzyme, in most cases due to mutations or deletions of the gene, whereas ultra-extensive metabolism (UEM) in general is caused by gene duplications.

The most important isoforms involved in drug metabolism are CYP2D6, CYP2C9, CYP2C19 and CYP3A4. Several of these CYP isoforms are known to be polymorphic which results in differential capacities in metabolising drugs such as omeprazole (proton pump inhibitors), phenytoin (anti-convulsant), verapamil (calcium-antagonists), propanolol (beta-blockers) and many others. The CYP2C9 isoform is involved in hydroxylation of tolbutamide, phenytoin and S-warfarin among others. Specifically, CYP2C9 converts S-warfarin into the inactive phenolic metabolite S-7-hydroxywarfarin and thereby controls the pharmacological activity of this drug. Here too, polymorphisms among these enzymes exist resulting in differential capacities to metabolise drugs. The genetic basis of this polymorphism is single nucleotide mutations resulting in the expression of two allelic variants, CYP2C9*2 and CYP2C9*3. The CYP2C9*2 allele has cysteine substituted for arginine at amino acid 144 in the protein and CYP2C9*3 has leucine substituted for isoleucine at position 359. The frequencies of these alleles have been reported to be between 7 and 19 % in Caucasian populations. Although homozygous individuals for these alleles are relatively uncommon, *in vitro* studies of the metabolism of warfarin have shown impaired catalytic ability by these variant proteins (Steward et al., Pharmacogenetics (1997) 7:361). For instance, CYP2C9*3 possesses only 5% of the catalytic capacity for S-warfarin as compared to the CYP2C9 wild type enzyme.

Warfarin is a widely used anticoagulant of coumarin type which acts by blocking synthesis of the vitamin K-dependant coagulation factors II, VII, IX and X in the liver. The indications for taking S-warfarin are all diseases where prevention of extensive blood clotting is a crucial factor in the efficient treatment of patients. Examples of such

diseases are acute embolic diseases of heart, lung or brain. In these cases the treatment is often combined with heparine. More specific indications are diseases where a lifelong treatment with anti-coagulants is required. Such diseases include recurrent venous thrombosis, pulmonary embolism and chronic atrial fibrillations. The major difficulties with the use of this drug are a broad range of interactions with other drugs as well as nutritional factors. The complicated treatment of patients with this drug carries the risk of serious hemorrhage in as much as 9 % per patient year (Fihn et al., Ann. Intern. Med. (1996) 124:970; Steward et al., Pharmacogenetics (1997) 7:361). Therefore, pretreatment evaluation of the CYP2C9 status of potential patients to be treated with warfarin would significantly reduce the risk of adverse drug reactions. Moreover, CYP2C9 metabolises the transformation of the anti-convulsant Valproic acid (VDA) into the unsaturated metabolite 4-ENE-VPA. 4_ENE-VPA acts hepatotoxic and causes several deaths yearly in the US (Sadeque et al., J. Pharmacol. Exp. Ther. (1997) 283:698).

The CYP2C19 isoform is involved in 4-hydroxylation (or 5-hydroxylation) of tricyclic antidepressants such as imipramin, anti-malarial prodrugs as for instance proguanil and proton pump inhibitors such as omeprazole or pentaprazole (Linder et al., Clinical Chemistry (1997) 43:254). This subfamily is polymorphogenic due to single nucleotide mutations (SNP) of the wild type allele. The M1 allele contains a G_{686}-A_{686} substitution which creates a novel aberrantly spliced CYP2C19 mRNA. This results in the production of an inactivated truncated protein. The M2 allele contains a G_{641}-A_{641} substitution resulting in a premature stop codon. Therefore, these two alleles represent poor metabolise phenotypes.

Detection of single-point mutations (SNP), such as the above mentioned mutations, can be performed using different techniques. In general, such assays can be subdivided into techniques where detection of SNP:s involves electrophoretic separation of DNA sequences and techniques using solid supports. Techniques using solid supports have several advantages as compared to electrophoretic separation techniques. Firstly, the solid-phase assays involve relatively few and simple manipulations that are amenable to full automation. Secondly, non-radioactive methods can conveniently be used in the solid

phase assays and thirdly, these assays give numerical results allowing e.g. statistical treatment.

As in the case of solid phase assays, different assay types may be distinguished. These techniques include hybridisation with sequence-specific oligonucleotide probes such as "reverse dot blot" or sandwhich hybridisation. These techniques require very careful design of the sequence-specific probes and close monitoring of reaction conditions and may thus only be performed in highly specialised laboratories. Similar problems are encountered with sequence-specific amplification which require careful optimisation of the PCR conditions. Here too, only highly specialised laboratories are capable of performing this technique. Finally, sequencing of defined DNA sequences requires a costly infrastructure and trained personnel which currently only can be provided at highly specialised laboratories.

Since the CYP2C19 and CYP2C9 enzymes metabolise a variety of drugs where relative overdosing poses a potential threat to the patient's health, there is need for a simple analytical test clarifying the genetic status of the individual prior to drug intake, as knowledge of a person's genetic status prior to drug intake could substantially reduce the risk for adverse drug reactions.

Summary of the invention

It has now turned out that it is possible carry out a simple test for measuring a patient's ability to metabolise a certain drug by applying a method comprising the steps of

a) isolating and/or providing detectable amounts of single-stranded DNA from said sample by using known methods;
b) hybridising the single-stranded DNA obtained in step a) with a detection primer comprising a plurality of nucleotide residues, said primer being complementary to a target nucleotide sequence immediately adjacent and 5' in relation to a defined point mutation of a single-stranded DNA encoding a cytochrome P450 isoform, where said point mutation is known to affect said isoform's ability to metabolise said drug, such

that there are no nucleotide residues between the defined point mutation and the 3' end of the detection primer that are identical to the first or second nucleotide residues of the point mutation to be detected, when the detection primer is hybridised to the target nucleic acid;

c) extending the primer using a polymerising agent in a mixture comprising one or more nucleoside triphosphates wherein the mixture includes at least one nucleoside triphosphate complementary to either the first or second nucleic residue comprising means for detecting the incorporation of the nucleoside triphosphate in a nucleic acid polymer, and optionally one or more chain terminating nucleoside triphosphates;

d) detecting the incorporation of the nucleoside triphosphate using said means, whereby it is determined whether said sample contains said point mutation of said cytochrome P450 isoform.

Detailed description of the invention

The solid phase mini-sequencing technique disclosed in WO91/13075 provides a cheap and robust assay which can be performed by any laboratory equipped with a thermal cycler. Moreover, this technique does not require any specially trained personnel. Furthermore, the solid phase mini-sequencing technique does not require radioactively labeled nucleotides. Therefor it exhibits higher safety standards than such techniques. Finally, this technique provides an excellent possibility of detecting either homozygote or heterozygote alleles within a defined sample.

Accordingly, the present invention relates to a method for determining the ability of cells in a sample to metabolise a certain drug comprising detecting a defined point mutation of a single-stranded DNA encoding a cytochrome P450 isoform, where said point mutation is known to affect said isoform's ability to metabolise said drug.

In another embodiment the present invention relates to detection primers useful in the above mentioned method, which primers hybridise to target nucleotide sequences

immediately adjacent and 5' in relation to a point mutation of a DNA, said DNA encoding an isoform of cytochrome P450.

In yet another embodiment, the present invention relates to a diagnostic kit for carrying out said method, said kit comprising at least one detection primer as defined above, at least two amplification primers derived from a sequence encoding a cytochrome P450 isoform , said amplification primers being chosen in such a way that a subsequence of said cytochrome P450-encoding sequence to which said detection primer hybridises is amplified, and a DNA-polymerising agent.

As disclosed herein, the term "drug" relates to drugs that are metabolised by cytochrome P450 isoforms. Examples of such drugs are omeprazole , pentaprazole, phenytoin, verapamil, propanolol, tolbutamide, S-warfarin, tricyclic antidepressants such as imipramin and anti-malarial prodrugs such as proguanil.

As disclosed herein, the term "detection primer" relates to an oligonucleotide which hybridises to a site immediately adjacent 5' in relation to a defined point mutation. The term "amplification primer" relates to one of two primers forming a primer pair that is used according to well-known amplification procedures such as PCR. Both detection primers and amplification primers according to the invention comprises 8 - 70 nucleotides, preferably 10-30 nucleotides, and most preferably 15 - 25 nucleotides.

As disclosed herein, the term "affinity pair" relates to a pair of chemical, preferably biochemical, compounds that binds specifically and strongly to each other. Examples of such pairs include, but are not limited to antibody-antigen, biotin-avidin/streptavidin, enzyme-substrate, a pair of complementary oligonucleotides, protein A-IgG, etc.

As disclosed here, the term "polymerising agent" relates to a DNA polymerising agent. An example of such an gent is the Klenow fragment of Escherichia coli DNA polymerase I, but any DNA polymerase can be used in the method of this invention.

According to the present invention, the presence of point mutations can be detected by adding labelled nucleotides to the detection primer. Any kind of detectable labels, such as one member of an affinity pair, radioactive nuclides, fluorescent compounds, enzymes inducing light emissions or colour changes etc. can be bound to an ordinary nuclotide in order to obtain a labelled nucleotide. Alternatively, it is possible to use modified nucleotides such as chain-terminating dideoxynucleotides. The skilled person is well aware of how to choose suitable labelled nucleotides as well as how to choose suitable detection procedures when carrying out the method according to the present invention.

The present invention will now be further described with reference to the enclosed figure ande tables, in which:

fig. 1 discloses a photo of an electrophoresis gel where lanes A-E represent the following PCR products: A: CYP2C9*2 (simplex PCR), B: CYP2C9*3 (simplex PCR), C: CYP2C9*2*3 (20 µl*3, multiplex PCR), D: CYP2C9*2*3 (15 µl * 3, multiplex PCR), E: CYP2C9*2*3 (10 µ * 3, multiplex PCR). In the multiplex PCR

constant primer concentrations for the CYP2C9*2 allele and decreasing concentrations of primers for the CYP2C9*3 allele were used in order to optimise the multiplex PCR conditions;

table 1 discloses results obtained when the PCR products shown in fig. 1 have been subjected to minisequencing reactions. Both specific and non-specific sequence primers as well as complimentary or non-complimentary nucleotides have been used. The figures shown in table 1 represent optical density (OD) values from an ELISA determined at 405 nm. The table shows which PCR products were coated onto the streptavidin-coated ELISA plate (columns), which sequence primers were used (rows) and which nucleotides were used in the sequencing reaction (columns);

table 2 shows the calculated ratio of the OD at 405 nm from nucleotides incorporated by the mini-sequencing reaction. The ratio values presented in this table have been

calculated from the OD values in table 1. The ratio was calculated as follows: *complementary nucleotide (OD at 405 nm/ complementary nucleotide + non-complementary nucleotide (OD at 405 nm).* A ratio of > 0.85 is significant for an incorporation of complementary nucleotides when using homozygous alleles.

Experimental procedures

The mini-sequencing technique is based on amplification of defined genes with PCR (Polymerase Chain Reaction) using biotinylated or otherwise conjugated oligonucleotides (primers). In general, where possible a multiplex amplification procedure is utilised. Following amplification, the biotinylated PCR products are immobilised on streptavidin-coated microwell plates and the PCR products are sequenced using an allele-specific oligonucleotide. Possible mutations within the immobilised PCR product representing a defined allele are detected by incorporation of a mutation-specific labelled nucleotide. Incorporatio of a complementary nucleotide can be detected either directly or indirectly utilising various established detection methods. Using this technique, it is possible to detect homozygote or heterozygote alleles based on single nucleotide mutations within an individual.

Genomic DNA can be prepared using any established method described in the literature (PCR Protocols, Innis MA et al., Academic Press 1990; PCR, a practical approach, McPherson, MJ et al., Oxford University Press, 1991) or using any DNA purification kit present. The DNA prepared in these experiments has been prepared using the QIAamp Blood Kit (Qiagen Inc, USA) according to the description provided by the manufacturer. Genomic DNA can be prepared using any sample-containing nucleated cells. The typical yield using the above mentioned DNA purification kit is 10 ng/µl. 250 ng of purified genomic DNA was used as a template in the subsequent PCR. The primers used for the PCR reactions are described in the sequence listing as SEQ.ID.Nos 4, 5, 7, 8, 10, 11, 13 and 14. In the following description of the experimental procedures for the PCR and mini-sequencing method of CYP genes, primers specificfor the CYP2C9 alleles were

used. The same experimental procedures as described below were used when CYP2C19 alleles were studied with the exception for using CYP2C19-specific oligonuclotides.

A 2x mastemix for the multiplex PCR of CYP2C9 alleles was prepared as follows:

- Tris-HCl (100 mM, pH8.8)
- $(NH_4)_2SO_4$ (30 mM)
- Triton X-100 (0.2 % vol./vol.)
- Gelatin (0.02% wt/vol.)
- dNTPs (0.4 µM)
- SEQ.ID.NO. 4, 5, 7, 8 (0.4 mM of each)
- $MgCl_2$ (3.0 mM)
- ddH_2O up to 500 µl

For the PCR reaction 50 µl of the above described 2x mastermix was subject to a PCR tube (thin wall PCR tubes, Perkin-Elmer Inc. USA). 24,5 µl of ddH2O, O,5 µl Taq-polymerase (2,5 Units, Perkin-Elmer, Inc, USA) and 25 µl geonomic DNA (250 ng) was added to the tube and the reaction mix was overlaid with 50 µl mineral oil.

The thermal conditions for amplification of the CYP2C9 alleles were as follows: An initial denaturation step at 96°C for 2 minutes thereafter 96°C (30 sec), 60°C (30 sec) (58°C for the CYP2C19 alleles) and 72°C (30 sec), 35 cycles. Following the PCR amplification, 100 µl of the amplified sample was mixed with 400 µl of Binding buffer (buffer 1) containing 20 mM sodium phosphate, pH 7.5, 100 mM NaC1 and 0.1 % (v/v) Tween-20.

50 µl aliquots were subsequently transferred to streptavidin coated said phase, such as microwell plates (MWP) which are commercially available (Labsystems, Helsinki, Finland). The MWP were then incubated at 22°C for 15 minutes. Following incubation, the immobilized PCR samples were denatured using a denaturing solution containing NaOH (50 mM) for 1 minute at 22°C. The MWP were washed using a buffer (buffer 2)

containing Tris-HC1 (40 mM, pH 8.8), EDTA (1mM), NaC1 (50 mM) and Tween-20 (0.1%).

For the minisequencing reaction, every well of the MWP was incubated with an appropriate minisequencing primer (final concentration 0.1 μM) diluted in 5 μl of 10 x DNA polymerase buffer (buffer 3) containing Tris-HCl (500 mM, pH 8.8), $(NH_4)_2SO_4$ (150 mM), $MgC1_2$ (15mM), Triton X-100 (1% V/V), Gelatin (0.1%W/V), DNA polymerase (0.1 units), flourescein-12-dNTP complementary to the nucleotide to be detected (final concentration of 0.1 μM) and ddH_2O to a final volume of 50 μl.

The MWP were incubated at 55° C for 30 minutes. Following the minisequencing reaction the MWP were washed using buffer 2. Incorporated nucleotides were detected using alkaline phosphatase (AP) conjugated anti-FITC monoclonal antibodies (0.75 U/ml) diluted in a buffer (buffer 4) containing : Hepes (25 mM), NaC1 (125 mM), $MgCl_2$ (2 mM), BSA (1%) and Tween-20 (0.3 % V/V). Incubation was done at 22° C for 15 minutes and the plates were subsequently washed using buffer 2. Detection of bound monoclonal antibodies was performed by incubation the MWP using a detection buffer (buffer 5) containing diethanolamine (10.6 % W/V), $MgC1_2$ (0.05 % W/V) and para-nitro-phenyl phosphate (4 mg/ml) for 20 minutes at 22° C. Detection of incorporated dNTP's was done at 405 nm using a commercially available spectrophotometer.

RESULTS

PCR amplification and minisequencing has been performed using CYP2C9 and CYP2C19 specific primers. The results shown below demonstrate amplification and minisequencing of the CYP2C9 alleles. Human genomic DNA was purified as described in the method section. 250 ng of genomic DNA was subjected to PCR as described above. The results of a representative experiment are demonstrated in figure 1.

Next a minsequencing reaction of the amplified DNA was done as decribed above. By using alleles specific sequencing primers incorporated dNTP's could be detected in subsequent detection steps as described above. These results are shown in table 1.

In order to obtain mumerical values a ratio of the OD was calculated based on the formula shown below. These results are demonstrated in table 2.

These results clearly show that by using gene-specific primers it was possible to amplify and sequence cytochrome P450 specific alleles using PCR and the minisequencing technique as described above.

TABLE 1

Sequence primers	CYP2C9*2 samples		CYP2C9*3 samples		*2*3 20 µl, 3 multiples		*2*3 15 µl, 3 multiples		*2*3 10 µl, 3 multiples		No coating	
CYP2C9*2 sek	2.211	C	0.186	G	0.697	C	0.702	C	0.615	C	0.137	C
CYP2C9*2 sek	0.157	U	0.161	U	0.191	U	0.201	U	0.312	U	0.138	U
CYP2C9*3 sek	0.151	C	0.191	G	0.198	G	0.192	G	0.239	G	0.139	G
CYP2C9*3 sek	0.164	U	1.220	U	0.694	U	0.818	U	0.697	U	0.132	U
CYP2C9*2 sek	0.186	G	0.141	C	0.179	A	0.222	A	0.161	A	0.139	A
CYP2C9*2 sek	0.153	A	0.147	A	0.183	A	0.224	A	0.167	A	0.140	A
CYP2C9*3 sek	0.164	G	0.153	C	0.265	A	0.302	A	0.200	A	0.137	A
CYP2C9*3 sek	0.147	A	0.336	A	0.253	A	0.283	A	0.223	A	0.129	A

TABLE 2

PCR Primers	Nucleotide	Ratio
CYP2C9*2	C	0.99
CYP2C9*3	U	0.97
CYP2C9*2*3 (20 µl)	C	0.95
CYP2C9*2*3 (20 µl)	U	0.95
CYP2C9*2*3 (15 µl)	C	0.92
CYP2C9*2*3 (15 µl)	U	0.94
CYP2C9*2*3 (10 µl)	C	0.75
CYP2C9*2*3 (10 µl)	U	0.77